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ORIGINAL PAPER

Overexpression of *MIC-3* indicates a direct role for the *MIC* gene family in mediating Upland cotton (*Gossypium hirsutum*) resistance to root-knot nematode (*Meloidogyne incognita*)

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Abstract

Key message **Transgene-based analysis of the MIC-3 gene provides the first report of a cotton gene having a direct role in mediating cotton resistance to root-knot nematode.**

Abstract Major quantitative trait loci have been mapped to Upland cotton (*Gossypium hirsutum* L.) chromosomes 11 and 14 that govern the highly resistant phenotype in response to infection by root-knot nematode (RKN; *Meloidogyne incognita*); however, nearly nothing is known regarding the underlying molecular determinants of this RKN-resistant phenotype. Multiple lines of circumstantial evidence have strongly suggested that the *MIC* (*M*eloidogyne *I*nduced *C*otton) gene family plays an integral role in mediating cotton resistance to RKN. In this report, we demonstrate that overexpression of *MIC-3* in the RKN-susceptible genetic background Coker 312 reduces RKN egg production by ca. 60–75 % compared to non-transgenic controls and transgene-null sibling lines. *MIC-3* transcript and protein overexpression were confirmed in root tissues of multiple independent transgenic lines with each line showing a similar level of increased resistance to RKN. In contrast to RKN fecundity, transgenic lines showed RKN-induced root galling similar to the susceptible controls. In addition, we determined that this effect of *MIC-3* overexpression was specific to RKN as no effect was observed on reniform nematode (*Rotylenchulus reniformis*) reproduction. Transgenic lines did not show obvious alterations in growth, morphology, flowering, or fiber quality traits. Gene expression analyses showed that *MIC-3* transcript levels in uninfected transgenic roots exceeded levels observed in RKN-infected roots of naturally resistant plants and that overexpression did not alter the regulation of native *MIC* genes in the genome. These results are the first report describing a direct role for a specific gene family in mediating cotton resistance to a plant-parasitic nematode.

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Introduction

Root-knot nematodes (RKN; *Meloidogyne* spp.) are obligate biotrophic pathogens of most vascular plants and have the dubious honor of being selected as the world's most damaging plant-parasitic nematode (Jones et al. 2013). Second-stage juveniles (J2) that hatch from eggs in the soil immediately seek out and penetrate host roots, preferably at the root tips, and migrate intercellularly through the root to the zone of differentiation where the feeding site is established (Williamson and Hussey 1996). Feeding sites formed by *Meloidogyne* spp. consist of multiple 'giant-cells' that serve as a nutrient sink in the plant and provide a constant supply of nutrients to the nematode as it matures and produces eggs (Williamson and Hussey 1996). Concomitant with giant-cell formation is the excessive swelling and multiplication of surrounding cortical cells which gives

rise to the characteristic root gall (Williamson and Hussey 1996).

While many important agricultural commodities serve as hosts for RKN, Upland cotton (*Gossypium hirsutum* L.) is particularly vulnerable to infection due to the absence of elite varieties with high levels of resistance. For example, during the 2013 growing season, RKN infection resulted in an estimated loss of 432,100 bales of cotton with an approximated value of $195 million (Lawrence et al. 2014). There have been only a few sources of RKN resistance identified in *G. hirsutum*, the most effective of which being originally released as Auburn 623 RNR and later incorporated into the M-series of germplasm lines (Shepherd 1974; Shepherd et al. 1996). Quantitative trait locus (QTL) mapping studies eventually concluded that this high level of RKN resistance was governed largely by two unlinked QTLs: one QTL located on chromosome 11 (Chr11) and linked to the SSR markers CIR316 and BNL1231 and another QTL located on Chr14 linked to the SSR markers BNL3545 and BNL3661 (Shen et al. 2006; Ynturi et al. 2006; Gutierrez et al. 2010; Jenkins et al. 2012). The presence of these QTLs and their genomic locations were later confirmed by He et al. (2014) using the RKN-resistant M-120 RNR germplasm line, a derivative of Auburn 623 RNR. The same mapping studies also indicated the two QTLs affected different aspects of the RKN-resistance phenotype. The Chr11 QTL more strongly influenced the severity of RKN-induced root galling, while the Chr14 QTL primarily impacted RKN egg production (Gutierrez et al. 2010; Jenkins et al. 2012; He et al. 2014).The RKN resistance mediated by the Chr11 and Chr14 QTLs appears to manifest as a two-stage process with an early effect on sedentary J2 at around 8 days after inoculation (DAI) and a later effect on nematode fecundity (Creech et al. 1995; Jenkins et al. 1995).

While significant advances have been made in determining the number and genomic location of loci that confer RKN resistance in *G. hirsutum*, the molecular underpinnings of the resistance phenotype remain unknown. An initial effort to identify differentially expressed proteins in immature/early galls of resistant and susceptible plants discovered a 14 kDa polypeptide that accumulated specifically in galls of resistant plants at 8 DAI (Callahan et al. 1997). Partial sequencing of the peptide led to the identification of the full-length cDNA which was named *MIC-3* (*M*eloidogyne *I*nduced *C*otton*3*) (Zhang et al. 2002). *MIC-3* was discovered to be only expressed in root tissues and no orthologous gene has been identified outside of the genus *Gossypium* (Zhang et al. 2002; Wubben et al. 2008). Strong circumstantial evidence that *MIC-3* is directly involved in RKN resistance was provided by measuring MIC protein and transcript levels in control and inoculated roots of resistant and susceptible plants. Callahan et al. (2004) showed that MIC protein levels in RKN-infected roots were significantly correlated with the galling index of the cotton variety. In other words, as the level of genetic resistance increased so did the level of MIC-3 protein in the infected root tissues (Callahan et al. 2004). A thorough examination of the *MIC-3* gene family revealed the existence of at least 15 *MIC*-like cDNAs that are expressed in cotton roots (Wubben et al. 2008). This study also demonstrated that RKN-resistant and -susceptible cotton varieties did not differ in their respective complements of predicted MIC peptides, but instead in the timing of peak *MIC* transcript accumulation in infected roots. RKN-resistant cotton roots reached peak *MIC* expression immediately prior to the first visible signs of resistance, i.e., delayed juvenile development and cessation in root gall growth (Wubben et al. 2008). In contrast, RKN-susceptible roots showed a delayed and much lower induction of *MIC* expression. These data strongly suggested to the authors that the *MIC* gene family most likely encoded a set of cotton-specific pathogenesis-related proteins (Wubben et al. 2008). Attempts to localize *MIC* family members to specific chromosomes in *G. hirsutum* succeeded in identifying six family members on the short arm of chromosome 4 and one family member on chromosome 19 (Buriev et al. 2010). In addition, a BLASTn search of the genome sequence of the extant D sub-genome representative, *G. raimondii*, failed to identify homologous *MIC* sequences within the interval defined by the homeologous Chr14 BNL3545 and BNL3661 marker loci (Wubben, unpublished data). In light of these findings, it is reasonable to hypothesize that *MIC-3* induction in resistant plants is an 'end-point' in the signaling cascade that is regulated by the RKN-resistance QTLs.

In this report, we describe the phenotypic characterization of a series of transgenic cotton lines that overexpress *MIC-3*. Transgenic cotton plants that accumulated high levels of MIC-3 transcript and protein in root and shoot tissues were developed from the RKN-susceptible obsolete cultivar Coker 312. We demonstrate that *MIC-3* overexpression reduces RKN reproduction by 60–75 % compared to non-transgenic Coker 312 plants; however, overexpression does not influence the severity of RKN-induced root galling nor does it alter susceptibility to the reniform nematode (*Rotylenchulus reniformis*). These results provide direct evidence that the *MIC* gene family is intimately involved in mediating cotton resistance to RKN.

Materials and methods

Creation of *MIC-3* overexpression construct

A genomic clone of the *MIC-3* open reading frame was PCR-amplified from genomic DNA of the cotton

germplasm line M-315 RNR (Shepherd et al. 1996) using forward and reverse primers that included BamHI and SacI restriction sites at their 5′-ends (forward primer: 5′-CATGGATCCATGGCTTGTCCTCCAACTCA-3′, reverse primer: 5′-GTAGAGCTCTTAATTGCAACCGCTCCACATG-3′). The PCR product was gel purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and ligated into the pDrive T/A vector using the Qiagen PCR Cloning Kit (Qiagen) per manufacturer's instructions. Multiple colonies were picked and sequenced to identify a clone that matched 100 % with the *MIC-3* genomic open reading frame (Accession No. AY072783). The *MIC-3* genomic clone was prepared as an insert for the pBI121 binary vector by digesting the plasmid first with SacI and then with BamHI (New England Biolabs, Ipswich, MA).

The pBI121 binary vector, kindly provided by Dr. Thomas Baum (Iowa State University), was heat shock transformed into DH10B competent cells (Invitrogen, Carlsbad, CA) and propagated. The ~2 Kb β-glucuronidase coding sequence downstream of the CaMV 35S promoter was removed by digestion with SacI followed by gel purification using the QIAEXII Gel Purification Kit (Qiagen). The linearized binary vector was then digested with BamHI and gel purified. The digested pBI121 vector and *MIC-3* insert were ligated using T4 DNA ligase and heat shock transformed into chemically competent DH10B cells. The correct orientation of the *MIC-3* insert was confirmed by PCR and sequencing.

Cotton transformation

The pBI121 binary vector containing the CaMV 35S::*MIC-3* construct was introduced into the EHA105 strain of *Agrobacterium tumefaciens* (Hood et al. 1993) by direct transformation as described by Walker-Peach and Velten (1984). The transformation protocol used hypocotyls cut from Coker 312 seedlings and submerged for 30 s at 28 °C in a diluted [1:19 in MSNH (4.4 g/L MS salts with Gamborg's B5 vitamins)] 24-hour-old culture of EHA105 containing the CaMV 35S::*MIC-3* pBI121 binary plasmid. The hypocotyl sections were then blotted dry on sterile filter paper to remove excess bacteria, transferred onto T2 plates (4.4 g/L MS salts with Gamborg's B5 vitamins + 0.453 μM 2,4-D and 2.325 μM kinetin + 30 g/L D-(+)-glucose + 2 g/L phytagel) and co-cultured for 2 days (28 °C, 16-h/8-h light/dark). The co-cultured hypocotyls were then blotted on sterile filter paper, transferred to MS2NK KM-CL plates (4.4 g/L MS salts with Gamborg's B5 vitamins + 2 g/L phytagel + 30 g/L D-(+)-glucose + 10.74 μM alpha-naphthaleneacetic acid + 0.465 μM kinetin + 266 mg/L cefotaxime + 50 mg/L kanamycin) to form callus tissue over a 12-week period (media changed every 3 weeks). At this stage, callus was cut from the hypocotyls and transferred to fresh MS2NK KM-1/4CL plates (4.4 g/L MS salts with Gamborg's B5 vitamins + 2 g/L phytagel + 30 g/L D-(+)-glucose + 10.74 μM alpha-naphthaleneacetic acid + 0.465 μM kinetin + 67 mg/L cefotaxime, + 50 mg/L kanamycin) for 3 weeks. The calluses were subsequently moved into liquid MSNH KM cell suspension medium (4.4 g/L MS salts with Gamborg's B5 vitamins + 30 g/L D-(+)-glucose + 50 mg/L kanamycin) and placed on a rotary shaker at 110 rpm for 7 days at 28 °C. Cultures were transferred to MSK KM plates (4.4 g/L MS salts with Gamborg's B5 vitamins + 2 g/L phytagel + 30 g/L D-(+)-glucose + 1.9 g/L KNO3, + 50 mg/L kanamycin) and placed in a 28 °C tissue culture room for embryo development.

DNA extraction and PCR

Genomic DNA was extracted from approximately 100 mg of fresh leaf tissue using the DNeasy Plant Mini-Kit (Qiagen) per manufacturer's instructions with modification as described in Gutierrez et al. (2010). Plants containing the overexpression construct were identified by PCR using a forward primer specific to the CaMV 35S promoter sequence (5′-GCCCAGCTATCTGTCACTTT-3′) and a reverse primer specific to the *MIC-3* coding sequence (5′-CTCTTAATTGCAACCGCTCCACATG-3′) at an annealing temperature of 55 °C. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

RNA extraction, reverse transcription and quantitative RT-PCR

Leaf tissues were collected from young seedlings, transferred to aluminum foil pouches, and flash frozen in liquid nitrogen. Roots of the same plants were gently washed free of soil and blotted dry with paper towels. Root tissues, minus the tap root, were transferred to aluminum foil pouches and flash frozen in liquid nitrogen. All tissues were stored at −70 °C until used for RNA extraction. Total RNA was isolated by the modified hot borate method as described by Wan and Wilkins (1994). Contaminating genomic DNA was removed using the DNA-*free*™ Kit (Ambion) per manufacturer's instructions. Nucleic acid quantitation was performed with a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). Reverse transcription was performed on 1 μg DNase-treated total RNA using the oligo d(T) primer supplied with the iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA) per the manufacturer's instructions.

Quantitative RT-PCR reactions were performed in triplicate in 96-well plates on a CFX96™ Real-Time System (Bio-Rad). Individual reactions consisted of 12.5 µL 2X iQ SYBR Green Super Mix, 0.625 µL 10 µM primer stock (forward/reverse combined), 10.25 µL water, and 1 µL template. Template cDNA consisted of a 1:10 dilution of the reverse transcription reaction. For standard curve generation, a dilution series of 1:5, 1:25, 1:125 and 1:625 of a reverse transcription reaction was used. Detection of native and transgene-derived *MIC-3* transcript was accomplished using the forward primer 5′-TTAGGGTT AAGTGGATTATTCTTTGG-3′ and the reverse primer 5′-TGGTTTTCGGTCGGAATGATCTTAGT-3′. Normalization of *MIC-3* expression was accomplished via levels of cotton α-tubulin expression in each sample (forward primer: 5′-GATCTCGCTGCCCTGGAA-3′, reverse primer: 5′-ACCAGACTCAGCGCCAACTT-3′). Normalization and fold-change expression calculations were performed using the CFX Manager Software package (Bio-Rad).

Protein isolation and western blotting

Total protein extraction from root and leaf tissues, immunoblotting, and MIC-3 protein detection were performed as described previously (Callahan et al. 2004). Equal loading between gel lanes was verified using the 2-D Quant Kit (GE Healthcare Bio-Sciences, Piscataway, NJ).

Nematode infection experiments

Root-knot nematode

A southern root-knot nematode (RKN; *Meloidogyne incognita*) race 3 population was maintained on kenaf (*Hibiscus cannabinus*) and cotton plants in the greenhouse. RKN eggs used in infection assays were collected from host plant roots via the method of Hussey and Barker (1973). Transgenic lines were evaluated in five independent greenhouse experiments with the following controls: Coker 312 (RKN-susceptible genetic background used for creating transgenic lines), M-8 (RKN-susceptible; Shepherd 1983) and M-240 RNR (RKN-resistant; Shepherd et al. 1996). Experimental units were comprised of 4–5 plants in a single row. Rows were ordered on a bench in the greenhouse in a completely randomized design in each experiment. Plant growth and egg inoculations were as described in Gutierrez et al. (2010). For data collection, individual plants were scored for root gall index while the plants comprising each row were grouped together for root fresh weight measurements and egg extraction as previously described (Gutierrez et al. 2010). Data were subjected to general linear model and analysis of variance using SAS version 9.3 (SAS Institute). Means were separated using Fisher's protected least significant difference (LSD) at $P \leq 0.05$.

The inoculum density experiment was conducted under growth chamber and greenhouse conditions. For both environments, cotton seedlings were pre-germinated and sown into 20 cm Cone-tainers containing a 1:3 soil mixture Wickham sandy loam soil:sand. A total of 24 experimental units [2 genotypes (Coker 312, transgenic line 14-7-1) × 4 inoculum levels (750, 1500, 2500, and 5000 eggs plant^{-1}) × 3 replicates] were randomly arranged within a 7 × 14 Cone-tainer rack for each environment. Inoculum solutions were prepared from a single concentrated solution of RKN eggs. Each experimental unit was comprised of three separate plants that were grouped together for root fresh weight measurements and egg extraction as described above. Seedlings assigned to the growth chamber were grown in a Percival PGC-9/2 chamber under 16-h light at 30 °C and 8-h dark at 25 °C. Statistical analysis was performed as described above.

Reniform nematode

A reniform nematode (*Rotylenchulus reniformis*) population was maintained on cotton plants in a greenhouse. Plant inoculations were performed using mixed vermiform life-stages that were collected according to Ganji et al. (2013). Cotton seedlings were grown in 500 cm^3 Solo Cups (Solo Cup Company, Lake Forest, IL) containing ca. 350 cm^3 of a 2:1 soil mixture of silty loam:sand. Solo Cups were inoculated with approximately 15,000 mixed vermiform life-stage nematodes on the day after planting with pre-germinated seedlings. Thirty days after inoculation, root systems were gently washed free of soil, the fresh root weight determined, and eggs extracted according to Hussey and Barker (1973). Eggs were counted in the same manner as for the RKN experiments. Statistical analyses were performed as described above.

Results

Development of transgenic cotton lines and confirmation of *MIC-3* overexpression

The RKN-susceptible obsolete cultivar Coker 312 was used for creating stable transgenic lines via *Agrobacterium tumefaciens* transformation that overexpress *MIC-3* (Accession No. AY072783). The β-glucuronidase coding sequence immediately downstream of the constitutive CaMV 35S promoter in the binary vector pBI121 was replaced with a *MIC-3* open reading frame that included the intron to prevent bacterial expression. The experimental pipeline used for identifying and characterizing the transgenic lines

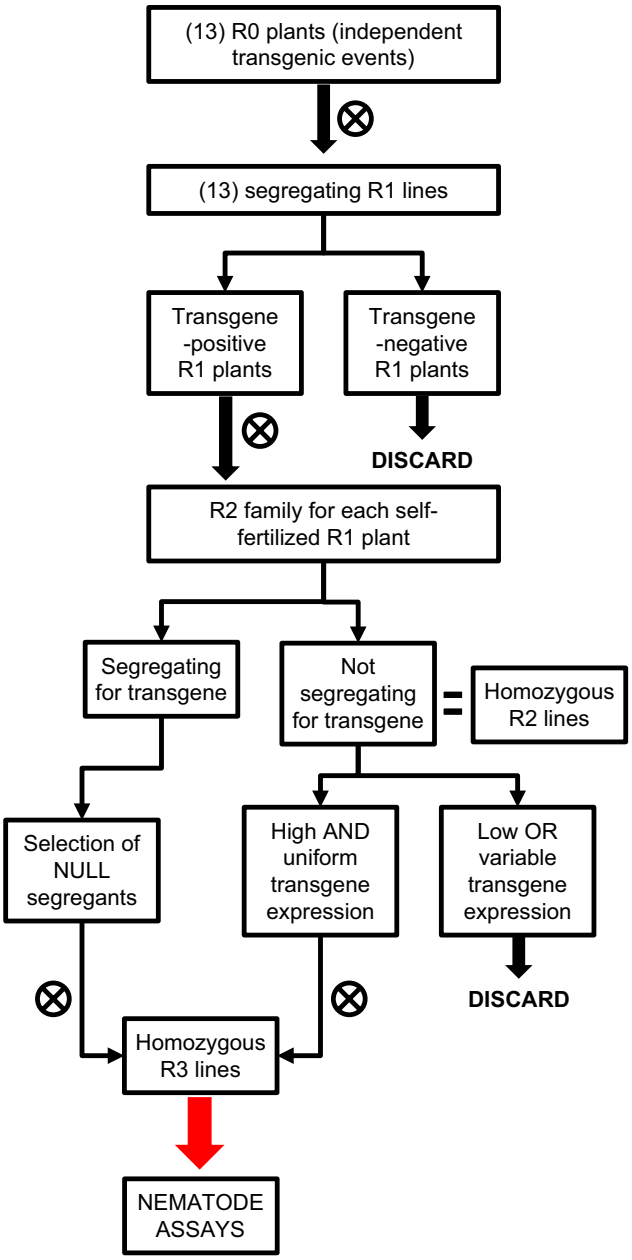

Fig. 1 Experimental pipeline for identifying and characterizing transgenic Upland cotton lines that overexpress *MIC-3*. ⊗: self-fertilization

is shown in Fig. 1. Thirteen primary transformants (R0 plants) were initially regenerated from *A. tumefaciens*-induced calli of which 10 carried the *MIC-3* overexpression transgene. The 10 transgene-positive R0 plants were self-fertilized to create R1 families that would segregate for the transgene. Transgene-positive individuals ($n = 3$–6) were identified within each R1 family by PCR and self-fertilized to create separate R2 families. Fifteen R2 plants within each family were PCR-screened for the presence/absence of the transgene to determine homozygosity of the parent plant. R2 families were considered to be homozygous if all plants were transgene positive. A total of 16 homozygous R2 families, representing seven independent transformation events, were selected for confirmation of *MIC-3* overexpression at the transcript and protein level. Southern blot analysis of the R2 families indicated the presence of a single copy of the transgene in each line (data not shown).

MIC-3 overexpression was measured at the transcript level in transgenic lines by quantitative RT-PCR (qRT-PCR). Total RNA was extracted from leaves and roots of multiple plants from each homozygous R2 family. In addition, RNA was extracted from leaf and root tissues of the non-transgenic lines Coker 312 and M-240 RNR (RKN-resistant). Total *MIC-3* transcript levels were measured using qRT-PCR primers that recognized transcript from both native *MIC-3* genes and from the overexpression construct. Multiple transgenic lines showed greatly elevated levels of total *MIC-3* transcript in both leaves and roots of which the most uniform over three biological samples are shown in Fig. 2a, b. *MIC-3* has been shown to be nearly undetectable in leaf tissue (Zhang et al. 2002; Wubben et al. 2008); consequently, the transgenic lines showed *MIC-3* leaf transcript levels that were approximately 60-fold to 160-fold greater than the non-transgenic controls (Fig. 2a). In root tissues, transgenic lines showed similar levels of *MIC-3* overexpression that were 7- to 14-fold higher than *MIC-3* transcript levels in non-transgenic control roots (Fig. 2b).

To verify that elevated *MIC-3* transcript levels translated to elevated MIC-3 protein, a Western blot was performed on total protein isolated from leaves and roots of transgenic lines and a non-transgenic control. The Western blot demonstrated that transgenic tissues accumulated MIC-3 protein as well as transcript and that these protein levels were roughly equivalent between transgenic lines (Fig. 2c).

MIC-3 overexpression reduces RKN fecundity

The RKN susceptibility of four independent transgenic lines (R3 generation; Fig. 1) was determined in replicated greenhouse experiments. Individual plants were scored for severity of root galling on a scale of 1–5 (1 = no galling). Roots from 4–5 plants of each entry were pooled, the fresh weight determined, and RKN eggs were collected. Results, shown in Fig. 3, demonstrate that *MIC-3* overexpression decreased RKN egg production ($P \leq 0.05$) compared to the Coker 312 and M8 non-transgenic controls (Fig. 3a). This reduction in RKN eggs g^{-1} root ranged from 60 to 75 %; however, none of the transgenic lines approached the level of resistance of the resistant control M-240 RNR which showed a 95 % reduction in eggs g^{-1} root (Fig. 3a). In contrast to egg production, the RKN gall indices of the transgenic lines were similar to those of Coker 312 and M8, while M-240 RNR showed reduced root galling (Fig. 3b).

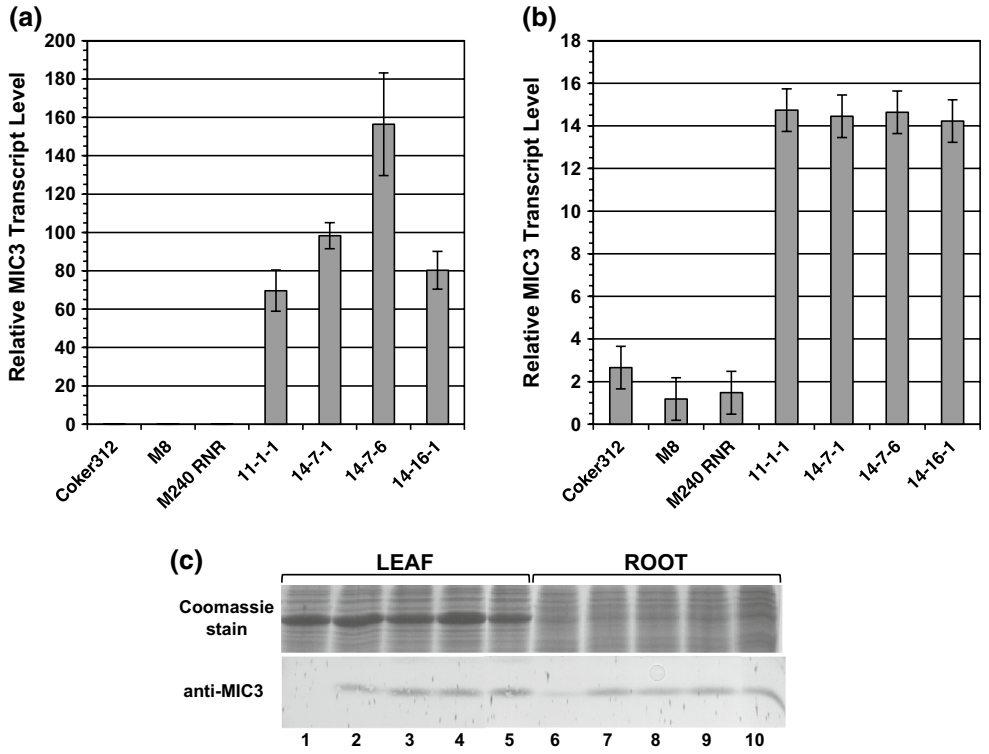

Fig. 2 *MIC-3* transcript and protein accumulation in cotton leaves and roots of overexpression lines. Total *MIC-3* transcript was quantified in leaves (**a**) and roots (**b**) of non-transgenic root-knot nematode (RKN)-susceptible (Coker 312, M8), non-transgenic RKN-resistant (M-240 RNR), and overexpression lines by quantitative real-time RT-PCR. Presented is the mean ± standard error of three biological replicates normalized to the expression of *Gossypium hirsutum* α-tubulin. **c** Western blot showing relative MIC-3 protein levels in leaves and roots of a non-transgenic control line (*lanes* 1,6) and transgenic lines 11-1-1 (*lanes* 2,7), 14-7-1 (*lanes* 3,8), 14-7-6 (*lanes* 4,9), and 14-16-1 (*lanes* 5,10)

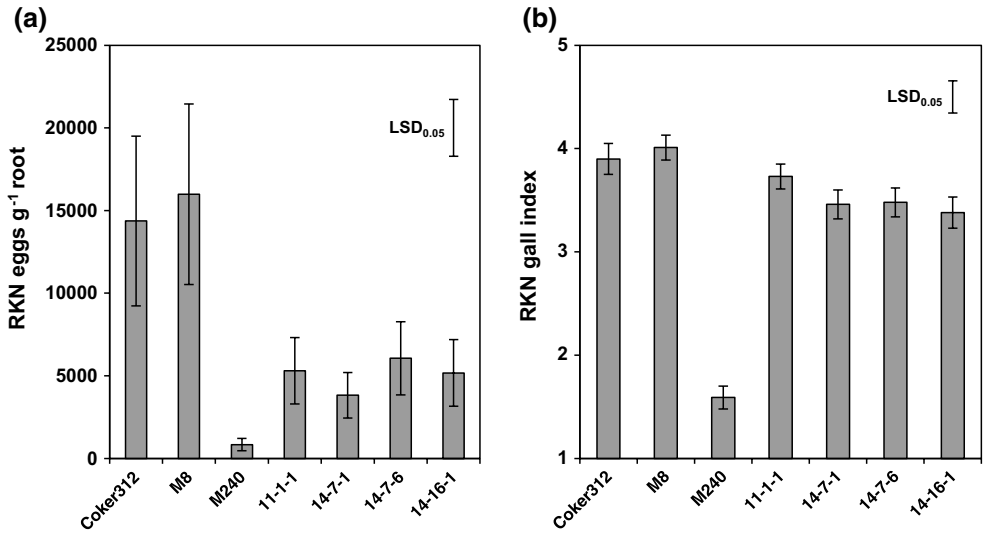

Fig. 3 Root-knot nematode (RKN) susceptibility of four independent *MIC-3* overexpression lines as measured by RKN eggs g^{-1} root (**a**) and galling index (**b**) compared to the RKN-susceptible controls Coker 312 and M8 and the RKN-resistant control M-240 RNR. Presented are the mean ± standard error and least significant difference (LSD) at $P \leq 0.05$ as determined by ANOVA using data combined from three separate experiments

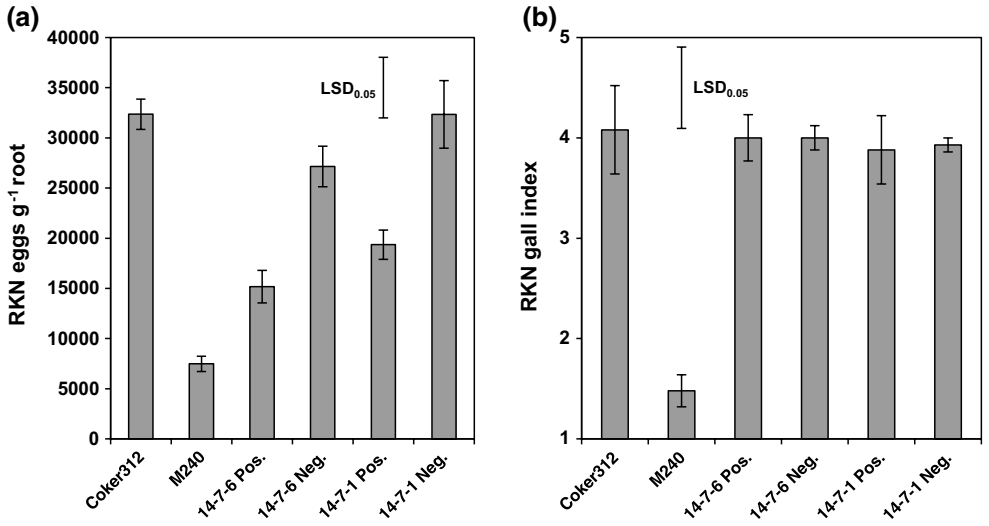

Fig. 4 Root-knot nematode (RKN) susceptibility of transgene-positive and transgene-null sibling lines of events 14-7-6 and 14-7-1 as measured by RKN eggs g^{-1} root (**a**) and galling index (**b**) compared to the non-transgenic controls Coker 312 (RKN-susceptible) and M-240 RNR (RKN-resistant). Presented are the mean ± standard error and least significant difference (LSD) at $P \leq 0.05$ as determined by ANOVA from one representative experiment

The RKN susceptibility of transgenic lines 14-7-1 and 14-7-6 was further evaluated in comparison to their respective transgene-null sib lines (Fig. 1). Unlike Coker 312 and M8, transgene-null segregants are genetically identical to their respective transgene-positive sibling lines except for the absence of the overexpression construct. For both the 14-7-1 and 14-7-6 lines, plants containing the *MIC-3* overexpression construct showed significantly less RKN egg production compared to their respective transgene-null sibling lines (Fig. 4a). The transgene-null plants showed RKN susceptibilities that were similar to that of the non-transgenic Coker 312. RKN gall indices remained unaffected by the presence or absence of the transgene as all tested lines, with the exception of M-240 RNR, showed gall indices of ca. 4.0 on the 1–5 scale (Fig. 4b).

Effect of initial inoculum density on line 14-7-1 resistance to RKN

To help determine the robustness of the resistance conferred by *MIC-3* overexpression, we assessed RKN reproduction on transgenic line 14-7-1 in comparison to Coker 312 over a range of initial inoculum levels in both growth chamber and greenhouse environments. We discovered that, regardless of environment, RKN reproduction on 14-7-1 and Coker 312 followed a similar trend as initial inoculum levels increased from 750 to 5,000 eggs $plant^{-1}$ (Fig. 5). Neither genotype showed a significant increase in final RKN reproduction at inoculum levels greater than 2,500 eggs/plant (Fig. 5). Transgenic line 14-7-1 showed less RKN eggs g^{-1} root at each level of inoculum compared to Coker 312; furthermore, when expressed as a percentage reduction relative to Coker 312, the level of resistance in 14-7-1 was relatively consistent across the inoculum levels (41.2–54.2 % reduction in the growth chamber and 23.4–45.6 % reduction in the greenhouse). These results demonstrate that *MIC-3* overexpression was not overwhelmed by higher levels of initial inoculum, but instead showed a consistent level of efficacy.

MIC-3 overexpression does not affect susceptibility to *Rotylenchulus reniformis*

Rotylenchulus reniformis is a sedentary semi-endoparasite that is often found together with RKN in the field and is itself an important cotton pest (Koenning et al. 2004). To determine whether *MIC-3* overexpression confers broad-spectrum resistance to sedentary nematode pathogens, we assessed the susceptibility of multiple transgenic lines to *R. reniformis*. As shown in Fig. 6, *MIC-3* overexpression does not confer increased resistance to the reniform nematode. This finding is in agreement with the observation that *R. reniformis* infection of RKN-resistant cotton plants does not induce *MIC-3* expression (Wubben et al. 2008). This finding also supports the conclusion that the observed decrease in RKN susceptibility is due to the presence of the transgene and not to an artifact from the transformation process.

Native *MIC* expression in cotton is root specific, however, the transgenic lines accumulated MIC-3 protein in both leaf and root tissues (Fig. 2c); therefore, the *MIC-3* overexpression lines were tested for their susceptibility to

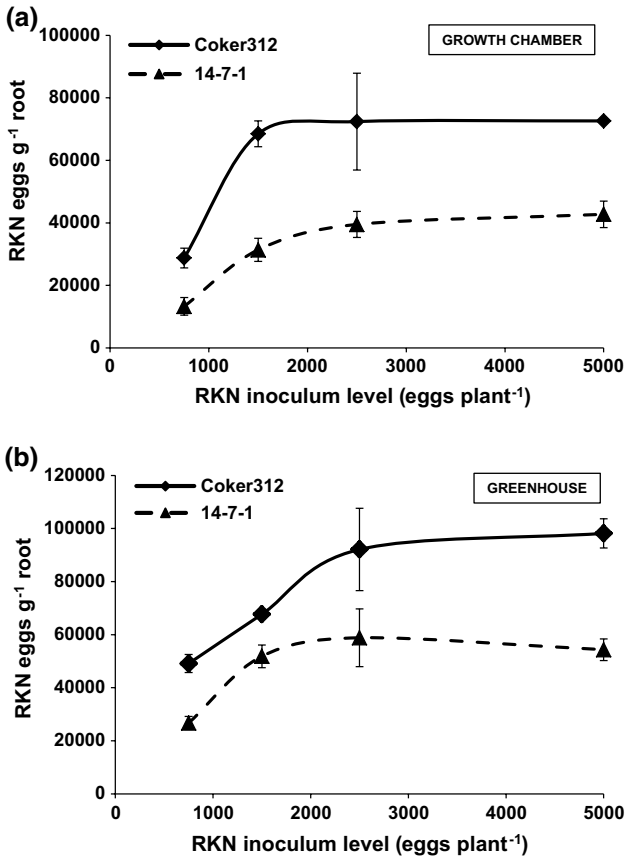

Fig. 5 Effect of inoculum level on final root-knot nematode (RKN) eggs g^{-1} root for the transgenic *MIC-3* overexpression line 14-7-1 and the non-transgenic susceptible control Coker 312. The experiment was performed in a growth chamber (**a**) and in the greenhouse (**b**). Presented are the mean ± standard errors from four replicates of one representative experiment

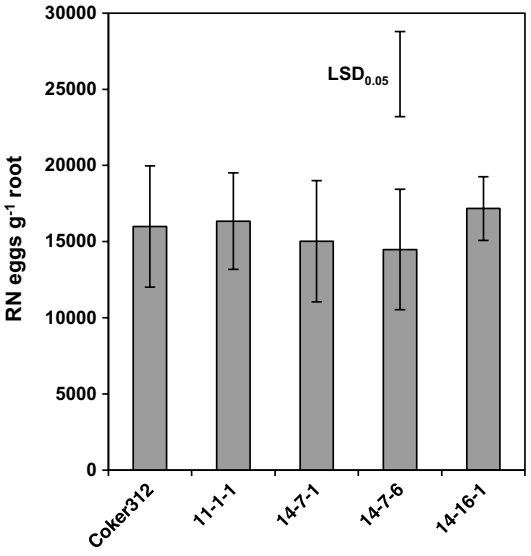

Fig. 6 Susceptibility of *MIC-3* overexpression lines to the reniform nematode, *Rotylenchulus reniformis*. Presented are the mean ± standard error and least significant difference (LSD) at $P \leq 0.05$ as determined by ANOVA from one representative experiment

the tobacco bud worm (*Heliothis virescens*), a foliar pest of cotton and other crops. We observed no change in larva survivability between the transgenic lines and susceptible checks (data not shown).

qRT-PCR analysis of *MIC-3* expression in control and transgenic lines after RKN infection

To determine whether *MIC-3* overexpression influenced the expression of native *MIC* genes, total *MIC-3* transcript levels were measured in transgenic lines and susceptible and resistant controls in response to RKN infection by qRT-PCR. Following RKN infection, total *MIC-3* transcript levels were not induced in Coker 312 or in the transgenic lines 11-1-1 and 14-7-1 (Fig. 7a). Only the RKN-resistant line M-315 RNR showed significant *MIC-3* induction as has been observed previously (Wubben et al. 2008). These results suggest that *MIC-3* overexpression does not lead to a state of heightened responsiveness of the native *MIC* genes in the transgenic lines, i.e., the transgenic lines behave in a similar fashion as the non-transgenic Coker 312.

We also wanted to verify that *MIC-3* transcript levels in transgenic roots were equivalent to or exceeded levels observed in RKN-infected resistant roots. A measurement of total *MIC-3* transcript levels in control roots and RKN-infected roots of the transgenic lines showed that absolute levels of *MIC-3* expression were much greater in the transgenic lines versus M-315 RNR (Fig. 7b). This result indicates that the level of *MIC-3* expression in RKN-infected transgenic roots exceeds that of infected resistant roots.

Discussion

Considerable effort has been put forth toward identifying the genetic positions of QTLs that mediate the high level of cotton resistance to RKN originally discovered by Shepherd (1974). These efforts were rewarded with the discovery of two major QTLs on chromosomes 11 and 14 that account for the majority of variation seen in the resistant phenotype (Gutierrez et al. 2010; Jenkins et al. 2012; He et al. 2014). In addition to QTL mapping, experiments were initiated with the goal of identifying specific gene products that function in RKN resistance. During the early stages of RKN infection, gall formation in resistant and susceptible plants is indistinguishable. This fact prompted researchers to investigate the protein complements of immature galls from the said plants in hopes of identifying a component of the early cotton resistance response to RKN. This endeavor

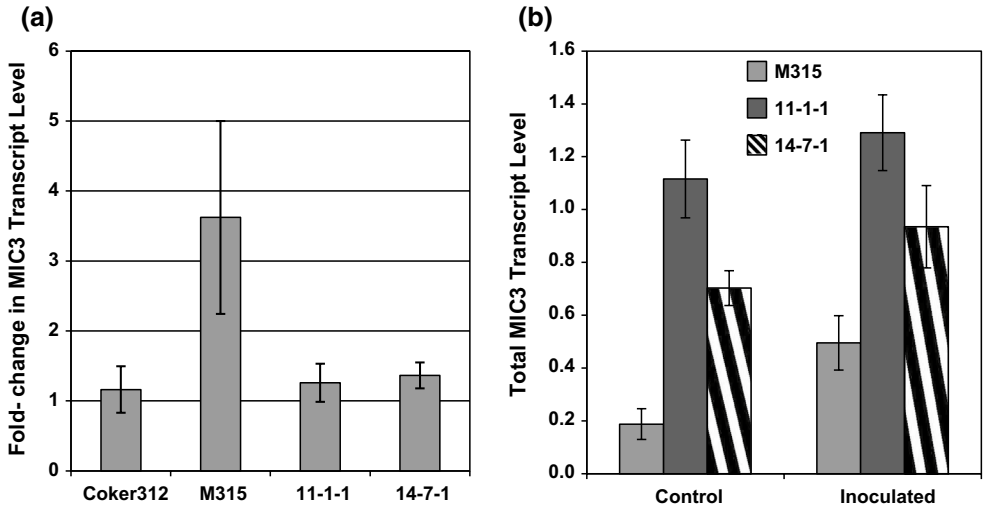

Fig. 7 Measurement of *MIC-3* transcript levels in transgenic and non-transgenic cotton roots following root-knot nematode (RKN) inoculation. **a** The fold-change in total *MIC-3* transcript level after RKN infection of Coker 312 (RKN-susceptible), M-315 (RKN-resistant), and overexpression lines 11-1-1 and 14-7-1. Presented is the mean ± standard error fold-change in *MIC-3* transcript levels (inoculated root/control root) from three biological replicates. **b** Total *MIC-3* transcript levels in control and inoculated roots of M315, 11-1-1, and 14-7-1. Presented is the mean ± standard error *MIC-3* transcript level from three biological samples. *MIC-3* transcript levels were normalized by the expression of α-tubulin

was successful with the isolation of the MIC-3 protein (Callahan et al. 1997; Zhang et al. 2002). While subsequent studies further supported the hypothesis that the *MIC* gene family was intimately involved in mediating cotton resistance to RKN (Callahan et al. 2004; Wubben et al. 2008), direct evidence for such a function was lacking.

In this report, we have provided direct evidence that the accumulation of MIC-3 protein in the root tissues of a RKN-susceptible genetic background is associated with dramatically decreased RKN egg production. This effect of *MIC-3* overexpression was highly repeatable and robust over multiple experiments and inoculum levels using transgenic lines recovered from several independent transformation events. At the molecular level, the mode of action of MIC-3 remains unknown despite numerous attempts by the authors to identify functional motif signatures within the peptide sequence (Zhang et al. 2002; Wubben et al. 2008). Complicating its characterization further is the fact that MIC-3 appears to be genus specific. Even with the current availability of multiple sequenced plant genomes, including that of *Theobroma cacao* (Argout et al. 2011) which is within the Malvaceae with *G. hirsutum*, no MIC-3 orthologs outside of the genus *Gossypium* have been identified.

We observed that *MIC-3* overexpression reduced RKN egg production in the absence of any discernible effect on the severity of RKN-induced root galling. Root galling is initiated early in the infection process and is a side-effect of giant-cell formation by the second-stage juvenile. The severity of root galling had been promoted as a simple means of evaluating cotton resistance to RKN (Zhang et al. 2006); however, it is now clearly established that high levels of RKN resistance are governed by QTLs that exert the majority of their influence on two separate aspects of the resistant phenotype. The resistance QTL localized to chromosome 11 most strongly affects the severity of RKN-induced root galling while the resistance QTL located on chromosome 14 negatively affects egg production with minimal impact on root galling (Shen et al. 2006; Ynturi et al. 2006; Gutierrez et al. 2010; Jenkins et al. 2012; He et al. 2014). When these QTLs are combined within a single genotype an extremely high level of resistance is observed (Gutierrez et al. 2010; Jenkins et al. 2012). This dichotomy in genetic control over the resistance phenotype agrees with earlier findings that RKN resistance appeared to be a two-stage process in cotton: an early effect at around 8 days after inoculation and a later effect at approximately 24 days after inoculation (Jenkins et al. 1995). It is currently unclear what roles the resistance QTLs on chromosome 11 and 14 play at these time points, but it is tempting to speculate, given their disparate effects on the resistance phenotype, that the QTLs act sequentially in the infection process. If so, because the QTL on chromosome 14 affects fecundity and not root galling, as we observed for the *MIC-3* transgenic lines, it may be that this QTL exerts its influence in part via the *MIC* gene family. Efforts are currently underway to test this hypothesis by measuring *MIC* gene expression in response to RKN infection in germplasm lines carrying different combinations of the resistance QTLs.

The molecular function of the *MIC* gene family, and of *MIC-3* specifically, remains a mystery. The absence of known functional motifs and domains precludes any conclusions regarding a possible cellular function based solely on sequence analysis; however, the timing of *MIC-3* expression in resistant plants was reminiscent of plant pathogenesis-related (PR) proteins (Wubben et al. 2008). Also, the fact that *MIC-3* is part of a large gene family in cotton that has clustered members throughout the genome supports the hypothesis that the *MIC* gene family represents a type of PR protein (Wubben et al. 2008; Buriev et al. 2010). The effect(s) of various PR proteins on plant-parasitic nematodes has been studied for some pathosystems. In *Arabidopsis thaliana*, overexpression of PR-1 decreased plant susceptibility to the sugar beet cyst nematode (*Heterodera schachtii*) and *M. incognita*, while overexpression of PR-3 reduced susceptibility only to *H. schachtii* and showed no effect on RKN (Hamamouch et al. 2011). The 'mode of action' of PR-1 against *H. schachtii* and *M. incognita* can only be speculated since PR-1, as in the case with MIC-3, does not contain any identifiable functional domains or motifs (Hamamouch et al. 2011). In another instance, a PR-10 protein with papain inhibitory activity was isolated from *Crotalaria pallida* (smooth rattlebox) roots that showed nematostatic and nematicidal activity when applied to *M. incognita* J2 (Andrade et al. 2010). The authors also demonstrated that the protein, with a size roughly equivalent to that of MIC-3 (14-15 kDa), could be taken up by the J2 and diffuse throughout the intestinal region (Andrade et al. 2010); therefore, it is possible that MIC-3 has a direct deleterious effect on the nematode as it may also be ingested during feeding. This mechanism would also help to explain the lack of effect of *MIC-3* overexpression on *R. reniformis* susceptibility. MIC-3 may be too large a peptide to be ingested by *R. reniformis*; however, the size exclusion limit of the *R. reniformis* stylet orifice would need to be more precisely determined before such a conclusion could be drawn.

While additional experimentation will need to be performed to show that MIC-3 is required for RKN resistance, e.g., silencing of *MIC* family members in a resistant line and observing increased susceptibility, the results presented here demonstrate that MIC-3 is at least sufficient for producing a moderately RKN-resistant phenotype. The molecular function and transcriptional regulation of *MIC-3* are additional avenues of research that we must continue to investigate if we are to fully understand the cotton–RKN-resistant interaction.

Author contributions MJW, FEC, and JNJ conceived of the research project. JV and JJB created the transgenic lines. MJW and FEC performed all transgenic line analyses. MJW wrote the manuscript.

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Conflict of interest The authors declare no conflict of interests.

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